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Melittin Enables Efficient Vesicular Escape and Enhanced Nuclear Access of Nonviral Gene Delivery Vectors*

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Entry of exogenously applied DNA into the cytoplasm and subsequent transport into the nucleus are major cellular barriers for nonviral gene delivery vectors. To overcome these barriers, we have covalently attached the cationic peptide melittin to poly(ethylenimine) (PEI). This conjugate condensed DNA into small, discrete particles (<100 nm in diameter), and the membrane lytic activity of melittin enabled efficient release of the DNA into the cytoplasm, as monitored by fluorescence microscopy and flow cytometry. Compared with PEI, the transfection activity was strongly increased within a broad range of cell lines and types tested, including different tumor cell lines but also primary hepatocytes and human umbilical vein endothelial cells. The early onset of gene expression (within 4 h, reaching maximal values after 12 h) and the high reporter gene expression achieved in slowly dividing or confluent cells suggested a further role of melittin after releasing the DNA into the cytoplasm. Intracytoplasmic microinjection of melittin-containing PEI-DNA complexes into fibroblasts produced 40% cellular frequency of reporter gene expression that was inhibitable by co-injection of wheat germ agglutinin, whereas simple PEI-DNA complexes showed only 10%. These data suggest that melittin enables release of nonviral gene transfer particles into the cytoplasm and also enhances their transport into the nucleus, possibly via the cationic cluster KRKR near the C terminus of the peptide.

Nonviral gene delivery systems generally exhibit a superior safety profile compared with viruses, and their use raises fewer ethical concerns; however, their relatively low efficiency of transgene expression is a major drawback. Poor release of the vectors from the endosome into the cytoplasm (1) and, subsequently, inefficient transfer into the nucleus (2) are both thought to contribute to their low transgene expression efficiency. The importance of endosomal escape can be shown using lysosomotropic drugs, such as chloroquine (3), or by incorporating membrane active peptides into the gene transfer complexes (4), which can lead to increases of >1000-fold in transfection. Similarly, inefficient nuclear transport was demonstrated when Pollard *et al.* (5) showed by microinjection that only 0.1–1% of free plasmids microinjected into the cytoplasm were transferred into the nucleus. The effects of the cell cycle on the efficiency of transfection using polyelectrolyte complexes was shown by Brunner *et al.* (6), compatible with the intact nuclear membrane restricting entry of DNA into the nucleus. Inefficient endosomal escape and poor nuclear transfer can both be addressed by incorporation of bioactive peptides into polyelectrolyte vectors. Nuclear localization sequences (NLS)[1] have been coupled either directly to the DNA (7–9) or to a DNA condensing lipid moiety (10). NLS peptides are mainly cationic, and they are easily incorporated into discrete polycation-DNA complexes. In contrast, amphipathic peptides described for pH-triggered lysis of the endosome membrane are negatively charged at neutral pH and can interact electrostatically with the positively charged complex surface (4), promoting formation of large aggregates (11). Such aggregates have a poorly defined interaction with cells *in vitro* and are unlikely to find useful applications *in vivo* (12).

A major objective of the present study was to use a cationic membrane-active peptide to enhance transfection activity of poly(ethyleneimine) (PEI)-DNA complexes, while avoiding electrostatic aggregation. Melittin, a cationic membrane lytic component of bee sting venom (13), was linked covalently to the cationic polymer PEI. Complexes formed by the conjugate with plasmid DNA are small and discrete, and mediate very high levels of transgene expression *in vitro* against a broad range of target cells. This activity is partially dependent on enhanced membrane lytic activity; however, melittin also enhances the post-endosomal activity of the complexes, increasing their nuclear accumulation. This nuclear targeting function may be mediated through the NLS-like sequence KRKR, contained within the melittin primary structure; hence, this simple oligopeptide appears to mediate two complementary functions during transfection.

EXPERIMENTAL PROCEDURES

Plasmids and Chemicals

The plasmids pGL3 (Promega, Southampton, United Kingdom (UK)) and pEGFP (CLONTECH, Basingstoke, UK) were purified with the Qiagen endo-free plasmid purification kit (Qiagen, Crawley, UK) and stored in water. Melittin (mel; GIGA VLKV LTTG LPAL ISWI KRKR QQ) and melittin-SH (mel-SH; CIGA VLKV LTTG LPAL ISWI KRKR QQ) were synthesized by Alta Biosciences (Birmingham, UK) to a purity >95%. Branched 25-kDa PEI (Aldrich, Poole, UK) was used as a 1 mg/ml working solution in water. Texas Red-X succinimidyl ester and YOYO-1 were from Molecular Probes (Leiden, The Netherlands). Cell

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[1] The abbreviations used are: NLS, nuclear localization sequence; PEI, (poly)ethylenimine; HBS, HEPES-buffered saline; FITC, fluorescein isothiocyanate; mel, melittin; BrdUrd, bromodeoxyuridine; HUVEC, human umbilical vein endothelial cell; FCS, fetal calf serum; PBS, phosphate-buffered saline; WGA, wheat germ agglutinin; EGFP, enhanced green fluorescent protein; PDP, pyridyldithio-propionate; TNBS, 2,4,6-trinitrobenzenesulfonic acid; DOTAP, N-(1-(2,3-dioleyloxy)propyl)-N,N,N-trimethylammonium salt.

culture reagents were from Life Technologies, Inc. (Paisley, UK). All other reagents were from Sigma (Poole, UK).

Conjugate Synthesis

Formation of Melittin-PEI (mel-PEI)—PEI (25 kDa, 600 nmol) were modified (1 h, room temperature) with 3-(2-pyridyldithio)propionic acid *N*-hydroxy-succinimide ester (9 µmol) in 200 mM HEPES, pH 7.4, purified by chromatography (Sephadex G-25 superfine HR 10/30 column; Amersham Pharmacia Biotech, Uppsala, Sweden) in 250 mM NaCl, 20 mM HEPES, pH 7.4. The PEI content was measured by TNBS assay (14), and the degree of modification with PDP determined by absorption at 343 nm after adding dithiothreitol to release the thiopyridone group (ε = 8080/M cm).

Melittin-SH (1240 nmol, in 300 µl of water) was conjugated with PEI-PDP (85 nmol, PEI/PDP 1/11 M/M) in 500 mM NaCl, 130 mM HEPES, pH 7.4 (2 h, room temperature under nitrogen), purified on a HR 5/5 MONO-S cation exchange column (Amersham Pharmacia Biotech; gradient elution 10–60% buffer A (buffer A, 5 M NaCl, 20 mM HEPES, pH 7.4; buffer B, 20 mM HEPES, pH 7.4)) and eluted at 2–2.5 M NaCl as monitored at 240 and 280 nm. The main fractions were pooled and concentrated/desalted on Centricon Plus-20 ultraconcentrators (Millipore, Bedford, MA; 8-kDa cut-off). A molar composition of PEI/melittin (1/10) was deduced from the change in the PEI/PDP molar ratio during the reaction, assuming that PDP can only react with the terminal cysteine of melittin. TNBS reactivity with melittin had no significant effect in this determination. Different batches of mel-PEI were prepared, resulting in molar ratios of PEI/melittin between 1/10 and 1/13.

Synthesis of Other Conjugates—Conjugation of PEI 25 kDa with human transferrin was carried out using 3-(2-pyridyldithio)propionic acid *N*-hydroxy-succinimide ester chemistry (15) and purified as described above (transferrin/PEI 0.6 M/1 M). Conjugation of PEI with Texas Red-X succinimidyl ester was carried out in 150 mM NaCl, 20 mM HEPES, pH 8.6, for 2 h at room temperature. The resulting conjugate was purified by Sephadex G-25 chromatography in HBS. Texas Red content was measured by absorption at 596 nm.

Erythrocyte Lysis Assay

Human erythrocytes were isolated from citrate-treated blood, washed in 150 mM NaCl, and resuspended at 10^7 cells/ml. Test materials were serially diluted 1:2 in 90 µl of buffer (140 mM NaCl, 20 mM phosphate, pH 7.4 or 5.5) using a V-bottom 96-well plate. 10 µl of erythrocyte suspension was added to each well, and the plate was incubated with constant shaking for 30 min at 37 °C. 100% lysis was achieved by adding 90 µl of water. After centrifugation at 300 × *g* for 10 min, released hemoglobin was determined at 490 nm (Microplate Autoreader, Bio-Tek Instruments, Montigny-le-Bretonneux, France).

Cell Culture Techniques

B16F10 cells (mouse melanoma), IGROV1 cells (human ovarian carcinoma), Rat-1 cells (rat fibroblast, ATCC CRL 2210), and HeLa cells (human cervical carcinoma, ATCC CCL-2) were grown in DMEM with Glutamax® and 10% heat-inactivated fetal calf serum (FCS). Primary human and rat hepatocytes were grown in the same medium in collagen-coated 48-well plates. Freshly isolated hepatocytes were allowed to attach for 4 h, washed with PBS, and used immediately. Human umbilical vein endothelial cells (HUVEC) were grown in M199 medium, 10% FCS, 2 mM glutamine/antibiotics with basic fibroblast growth factor (20 ng/ml; kindly provided by Selective Genetics, San Diego, CA). K562 cells (human chronic myelogenous leukemia; ATCC CCL-243 K-562) were cultured in Iscove's modified DMEM, 10% FCS, 2 mM glutamine.

Complex Formation

DNA was condensed with polycation in either HEPES (10 mM, pH 7.4) or HBS (150 mM NaCl, 20 mM HEPES, pH 7.4) with a final DNA concentration of 20 or 40 µg/ml (11). The N/P ratio represents the molar ratio of nitrogen in PEI to phosphate in DNA.

Transmission Electron Microscopy

(mel)-PEI-DNA complexes (N/P 5, mixed in 10 mM HEPES, pH 7.4) were applied onto mica, air-dried, and stained with uranyl-acetate. Dried samples were then rotary-shadowed with platinum and viewed on a model 1200 EX electron microscope (Jeol, Tokyo, Japan).

Photon Correlation Spectroscopy

A Zetasizer 1000 (Malvern, UK) was used for size determination of complexes with 10 subruns conducted for each measurement. Results were analyzed using Contin software.

Flow Cytometry

Cells were analyzed on a Coulter Epics XL flow cytometer equipped with a 488 nm argon laser. Fluorescence was detected using 520 nm (FL1) and 575 nm (FL2) bandpass filters for EGFP. Background fluorescence and autofluorescence were determined using mock-treated cells. Cellular debris showing reduced side and forward scatter was excluded from further analysis. Approximately 10,000 events were acquired per sample. Data analysis was carried out using the program WinMDI (by Joseph Trotter; available at facs.scripps.edu).

Fluorescence Microscopy

DNA was incubated with YOYO-1 (1 dye molecule/300 bp) (16) and then condensed with polycation (PEI or mel-PEI) containing 10% Texas Red-PEI. Cells were incubated with complexes in DMEM/FCS for 4 h at 37 °C and washed with PBS, and medium was replaced with phenol red-free DMEM/FCS. Cells were viewed on a Zeiss Axiovert-100 inverted microscope with heated stage. Fluorescence was viewed with filter sets appropriate for fluorescein or Texas Red, pictures were taken with a CCD camera (Princeton Instruments, Marlow, UK).

Analysis of Intracellular Processing of the Fluid Phase Marker FITC-Dextran

Cells were incubated with 1 mg/ml FITC-dextran (38 kDa) and complexes (N/P 5) in DMEM/FCS for 2 h at 37 °C, washed with PBS, and incubated for another 2 h without FITC-dextran. Cells were then washed with PBS, trypsinized, distributed into two cytometer tubes, and stored on ice. Monensin was added to one tube (1 µg/ml) and incubated on ice for 10 min. Flow cytometry data were acquired in logarithmic mode and analyzed in linear mode.

Transfection Procedures and Measurement of Cell Uptake

Complexes were added to adherent cells in 75 µl (48-well plates) or 300 µl (24- and 12-well plates) of FCS-containing medium, or to K562 cells (96-well plates, 50,000 cells/well) in 50 µl of medium. After 4 h complexes were removed and replaced by fresh medium. Gene expression was measured after 24 h. For luciferase detection, cells were washed once with PBS, lysed in Promega cell lysis solution, and assayed as described recently (17). 2 ng of recombinant luciferase (Promega) corresponded to 10^7 light units. Protein content was measured using the Bio-Rad assay (Bio-Rad Laboratories, Hemel Hempstead, UK). EGFP expression was analyzed by diagonal gating (11).

For radiolabeling experiments, DNA was spiked with ^{32}P-labeled DNA (18). B16F10 cells (80,000 cells/well in 48-well plates) in 75 µl of DMEM/FCS were incubated with 12.5 µl of complex solution containing 250 ng of DNA (N/P 5, HEPES). After 4 h cells were washed with PBS and lysed in Promega cell lysis solution, and geometry-corrected analysis for the contained radioactivity was conducted in a Packard scintillation counter. Transfections have been carried out in several independent experiments, and representative data are shown.

Measurement of Bromodeoxyuridine Incorporation

Cells were incubated in 10 µM bromodeoxyuridine (BrdUrd; Roche Diagnostics, Lewes, UK) for 6 h, harvested after 24 h, fixed in ethanol, and treated according to the manufacturer's recommendations. Staining with antibodies was carried out in 0.1% bovine serum albumin, 0.005% Triton X-100, PBS at room temperature (monoclonal mouse anti-BrdUrd (1:100, Roche), goat anti-mouse Alexa-488 (1:500, Molecular Probes)).

Microinjection into Rat-1 Fibroblasts

Microinjection was performed using an Eppendorf microinjection system (17). Tetramethylrhodamine-dextran (150 kDa) was added to the complexes (N/P 5, 20 µg/ml DNA) at a final concentration of 2 mg/ml. In some studies tetramethylrhodamine-labeled wheat germ agglutinin (WGA, Sigma) was added. To prevent aggregation of the complexes by the lectin, bovine serum albumin (final concentration 5 mg/ml) was added prior to the addition of WGA.

RESULTS

Biophysical Properties of PEI-DNA and mel-PEI-DNA—The membrane lytic activity of melittin was checked following its conjugation to PEI using a erythrocyte lysis assay. mel-SH and mel-PEI conjugate both showed erythrolysis at pH 7.4 (50% lysis occurred at 2–4 µM melittin content in each case) and at pH 5.5 (50% lysis at 4–8 µM melittin content for mel-SH, 2–4

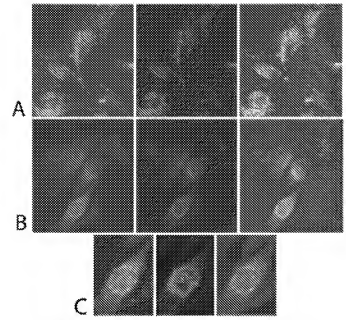

FIG. 1. **Intracellular fate of polyplexes ± melittin.** B16F10 cells (25,000 cells/well, 24-well plate) were incubated with 50 μl of YOYO-1-labeled complexes (N/P 5, generated in HEPES in 300 μl of medium. Fluorescence microscopy of cells incubated either with PEI-DNA (*A*) or mel-PEI-DNA (*B* and *C*) is shown. *left*, YOYO-1 fluorescence; *center*, Texas Red fluorescence; *right*, overlay.

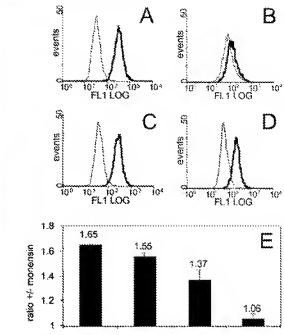

FIG. 2. **Measurement of endosomal release.** B16F10 cells (25,000 cells/well, 24-well plate) were incubated with 300 μl of DMEM containing FITC-dextran alone (*A*), in the presence of 100 μM chloroquine (*B*), plus 50 μl of PEI-DNA complex (*C*), or plus 50 μl of mel-PEI-DNA (*D*). Cells were harvested and analyzed by flow cytometry as described under "Experimental Procedures." *Narrow line*, without monensin; *bold line*, with monensin. *E*, the mean fluorescence of cells in the presence of monensin was divided by the mean fluorescence of the corresponding cells in the absence of the drug. *Sample 1*, FITC-dextran alone; *sample 2*, + 50 μl of PEI-DNA; *sample 3*, + 50 μl of mel-PEI-DNA; *sample 4*, + 100 μM chloroquine. Average values shown are obtained from at least two independent experiments ± S.D.

μM for mel-PEI). PEI alone did not lyse erythrocytes. Electron microscopy showed that mel-PEI condensed DNA into spherical polyplexes with diameter 32 ± 8 nm, similar to DNA-PEI complexes. Laser light scattering showed that mel-PEI-DNA complexes had diameter of 73 ± 1 nm (polydispersity 0.125), compared with 67 ± 1 nm (polydispersity 0.119) for PEI-DNA.

Intracellular Behavior of PEI-DNA Complexes Containing Melittin—Cell association of PEI-DNA and mel-PEI-DNA complexes was measured using both YOYO-1-labeled DNA with analysis by flow cytometry and ^{32}P-labeled DNA, analyzed by scintillation counting. In both cases the uptake of complexes was unaffected by the presence of melittin in the complex (data not shown). To examine the intracellular distribution, B16F10 cells were incubated with fluorescently labeled PEI-DNA or mel-PEI-DNA complexes, washed, and viewed live to rule out fixation artifacts. After 4 h of incubation, PEI-DNA complexes (N/P 5) appeared in a punctate intracellular pattern, suggesting entrapment within vesicles (Fig. 1*A*). In contrast, mel-PEI-DNA complexes (N/P 5) showed fluorescence dispersed throughout the cytoplasm and also the nucleus (Fig. 1, *B* and magnified image in *C*). YOYO-1-labeled DNA and Texas Red-PEI showed co-incident distributions in both cases, suggesting the complexes remained intact. The cytoplasmic fluorescence of the melittin-containing complexes suggests that melittin may facilitate release of polyplexes into the cytoplasm, probably by lysis of intracellular vesicles. To check whether the effect could be caused by the influence of melittin on charge properties, PEI-DNA complexes were evaluated across a range of N:P ratios (up to 9, data not shown); however, none produced a fluorescence distribution similar to mel-PEI-DNA.

We attempted to quantify the supposed endosomolytic action of melittin by incubating cells with complexes in the presence of FITC-labeled dextran, a fluid phase marker that co-internalizes into endocytic vesicles (Fig. 2). Acidification of endosomes by the ATP-dependent proton pump leads to partial quenching of FITC-dextran fluorescence, whereas the fluorescence of any FITC-dextran that enters into neutral compartments (*e.g.* the cytoplasm) should be unquenched (19). Cells were harvested and analyzed by flow cytometry either in the presence or absence of monensin, a drug known to discharge intracellular pH gradients and restore quenched FITC fluorescence (20). Cells incubated with FITC-dextran alone exhibited much greater fluorescence when analyzed in the presence of monensin (Fig. 2*A*), suggesting the presence of FITC-dextran within acidified endosomes or lysosomes. The presence of chloroquine in the incubation medium effectively removed this ability of monensin to enhance fluorescence (Fig. 2*B*), compatible with the lysosomotropic action of chloroquine leading to buffering of pH in the FITC-dextran-containing vesicles (3). When cells were incubated with PEI-DNA complexes in the presence of FITC-dextran, monensin again showed significant ability to enhance FITC fluorescence (Fig. 2*C*); this suggests the FITC-dextran is entering acidified vesicles, and therefore the buffering effect of PEI is not adequate to halt endosomal acidification completely. In contrast, when cells were incubated with mel-PEI-DNA in the presence of FITC-dextran, monensin exerted only a slight enhancement of FITC fluorescence (Fig. 2*D*), compatible with the FITC-dextran being largely present in a nonacidified compartment, such as the cytoplasm.

To normalize these data, the mean fractional increase in fluorescence mediated by monensin was calculated for each treatment (21) (Fig. 2*E*). Compared with the untreated FITC-dextran control (*sample 1*), co-incubation with mel-PEI-DNA complexes significantly decreased the ability of monensin to enhance FITC fluorescence (*sample 3*), whereas the corresponding PEI-DNA complexes showed substantially less activity (*sample 2*). Co-incubation with chloroquine resulted in a ratio of close to 1 (*sample 4*), as the lysosomotropic drug efficiently buffers endosomal/lysosomal vesicles (see above). The

TABLE I
Cell transfections

For luciferase gene expression, B16F10, IGROV1, HeLa, Rat-1, HUVEC p2–FGF, rat and human hepatocytes (8000, 10,000, 25,000, 25,000, 50,000, and 15,000/well, respectively) were transfected with 250 ng (nil N/P 5 except rat hepatocytes N/P 3.5). HUVEC 4 and 5 and K562 cells (50,000/well) with 500 ng of DNA (N/P 5). DOTAP–DNA complexes were prepared with a DOTAP/DNA ratio of 4/1 w/w in HBS *. complexes generated in HBS. **, transferrin targeted complexes. For EGFP transfection, B16F10 cells were transfected as described for luciferase. HeLa, IGROV1, and HUVEC p4 and p5 (30,000 cells/well) were transfected in 24-well plates with 1 μg (HeLa, IGROV1) or 2 μg (HUVEC) of DNA (N/P 5). FGF, fibroblast growth factor; p, passage number.

Cell line	Luciferase			EGFP-positive cells		
	PEI-DNA	mel-PEI-DNA	DOTAP-DNA	PEI-DNA	mel-PEI-DNA	DOTAP-DNA
	RLU × 10^3/mg protein			%		
B16F10	1432 ± 185	98,060 ± 12,388		13.0 ± 4.0	77.4 ± 2.2	
B16F10*	77,809 ± 16,587	73,479 ± 8114	15,469 ± 1626	21.5 ± 4.6	60.2 ± 1	
IGROV1	34 ± 2	2980 ± 119		1.6 ± 0.24	21.8 ± 0.25	
IGROV1*	59 ± 13	8596 ± 2081		0.14 ± 0.01	55.8 ± 1	0.9 ± 0.2
HeLa	74 ± 8.7	931 ± 263		0.91 ± 0.07	24.6 ± 1.3	
HeLa*		1797 ± 343	522 ± 109	0.59 ± 0.17	51.8 ± 1.1	6.6 ± 0.5
K562**	87 ± 21	344 ± 44				
K562	20 ± 16	86 ± 6.6				
Rat-1*	21 ± 8	181 ± 53	157 ± 5			
HUVEC p4 + FGF	0.8 ± 0.37	30.9 ± 11.7	16.6 ± 4		28.0 ± 0.87	1.6 ± 0.27
HUVEC p5 + FGF		36.2 ± 2.9		0.04 ± 0.01	8.19 ± 1.7	0.34 ± 0.33
HUVEC p2 + FGF*	0	1.2 ± 0.3				
Primary rat hepatocytes	1.2 ± 1.5	43 ± 25				
Primary human hepatocytes*	0	3.66 ± 0.45	0.94 ± 1.09			

effect of melittin is thought unlikely to reflect pH buffering, because melittin contained in the complexes does not exhibit significant buffering capacity. The possibility that melittin acts to transfer FITC-dextran into the cytoplasm is therefore more plausible and fits well with the fluorescent images described earlier (Fig. 1).

Increased Gene Expression of mel-PEI-DNA Complexes—The transfection activity of mel-PEI was assessed using several different cell types, with both luciferase and EGFP reporter genes (Table I). Activity was compared with that of DNA complexed with unmodified PEI or with the cationic lipid (DOTAP), with serum present throughout. The mel-PEI-DNA complexes invariably showed much greater activity than PEI-DNA (up to 700-fold for luciferase, over 20-fold for EGFP). Simple mixture of PEI and mel-SH prior to complexation with DNA did not increase transgene expression (data not shown). When applied to the human ovarian carcinoma cell line IGROV1, PEI-DNA complexes showed less than 2% transfection. This low level of activity is in accord with observations by Poulain and colleagues (22), who found very low transfection efficiency of IGROV1 cells with branched 25-kDa PEI. We verified their results (data not shown) and found that melittin also shows promising activity on even this cell line, with mel-PEI-DNA achieving over 20% EGFP positivity. Similar effects were also found using HeLa cells and early passage HUVEC; whereas PEI-DNA transfection achieves only about 1% transfected cells, mel-PEI-DNA complexes reproducibly achieved transfection rates of up to 30%. When transfecting nondividing primary rat hepatocytes or early passages of HUVEC cultured under minimal conditions (very slowly dividing), again melittin showed significant enhancement of transfection against a very low background achieved by PEI-DNA. The possibility of combining the activity of melittin with active targeting was demonstrated in K562 cells. Both mel-PEI-DNA and transferrin-targeted mel-PEI-DNA complexes were approximately 4-fold more efficient compared with their melittin-free counterparts, suggesting that melittin enhancement of transfection can be combined with active targeting.

The effect of melittin was also evaluated using complexes that were caused to aggregate by producing them in HBS. Such PEI-DNA aggregates usually show a 10–1000-fold higher gene expression level *in vitro* compared with their nonaggregated PEI-DNA counterparts, generated under low ionic conditions (6, 11, 23), thought to reflect increased levels of cellular association and improved endosomal escape because of their greater "proton sponge" properties (24). Whereas there was a clear increase in gene expression on B16F10 cells using aggregated PEI-DNA complexes, this effect could not be found on IGROV1 or HeLa cells. In contrast, with aggregated mel-PEI-DNA complexes, gene expression was further increased in the latter cases.

Time Course of Transgene Expression—The time course of transgene expression using mel-PEI-DNA was compared with PEI-DNA, with both small and HBS-aggregated formulations of each. With PEI-DNA complexes, initial transgene expression could be detected after 8 h (large aggregates) or 12 h (small complexes) and expression reached its peak 24 h following transfection. In contrast, both formulations of mel-PEI-DNA showed much faster transgene expression, detectable after just 4 h, and maximum levels achieved by 12 h (Fig. 3). Recent studies have suggested that active nuclear transport of DNA leads to a noticeably earlier onset of transgene expression (8), and this raised the possibility that melittin might exert a post-endosomal function, possibly enhancing transfer of the DNA into the nucleus.

Influence of Cell Density and Cycling Status on the Efficiency of Transfection—To probe the usefulness of melittin in overcoming intracellular barriers others than endosomal release, we evaluated its transfection-enhancing effects in either fast growing B16F10 cells (grown at low density) or confluent cells with reduced cycling activity (Fig. 4). Rapidly dividing cells (8000 cells/well, 71 ± 2% BrdUrd-positive) always showed greater susceptibility to transfection than confluent cells (80,000 cells/well, 39 ± 5% BrdUrd-positive), and mel-PEI-DNA complexes showed better activity than PEI-DNA. The endosomolytic drug chloroquine increased transgene expression in rapidly dividing cells with PEI-DNA but not mel-PEI-DNA, perhaps suggesting that endosomal escape restricts expression of PEI-DNA in these cells, whereas the presence of melittin removes this barrier. Surprisingly, using confluent cells chloroquine did not enhance transgene expression with PEI-DNA, although it did potentiate mel-PEI-DNA. This could suggest that other factors in confluent cells, such as decreased nuclear membrane permeability, might restrict the activity of PEI-DNA conjugates more than the activity of mel-PEI-DNA conjugates.

Microinjection into Rat-1 Fibroblasts—Microinjection studies were performed using Rat-1 fibroblasts with plasmid encoding EGFP (Fig. 5). Only cells exhibiting tetramethylrhodamine-

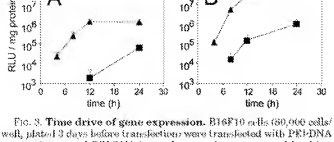

FIG. 3. **Time drive of gene expression.** B16F10 cells (60,000 cells/well, plated 2 days before transfection) were transfected with PEI-DNA (*rectangles*) or mel-PEI-DNA (*triangles*) complexes generated in either HEPES (*A*) or HBS (*B*) for 4 h (N/P 5). At the indicated time points, cells were harvested and analyzed for luciferase gene expression

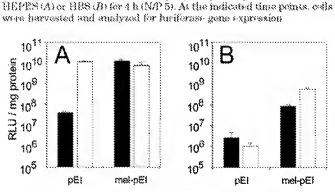

FIG. 4. **Influence of mitotic activity on reporter gene expression.** B16F10 cells were transfected with complexes N/P 5 generated in HEPES. Logarithmically growing (8000 cells/well plated the day before) (*A*) or confluent cells (80,000 cells/well plated 3 days before transfection) (*B*) were transfected either in the absence (*full bars*) or presence of 100 μM chloroquine (*open bars*) during the 4-h incubation period. After 24 h cells were harvested and analyzed for luciferase gene expression.

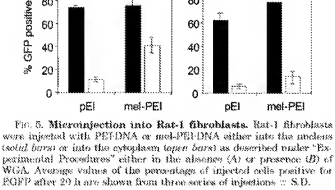

FIG. 5. **Microinjection into Rat-1 fibroblasts.** Rat-1 fibroblasts were injected with PEI-DNA or mel-PEI-DNA either into the nucleus (*solid bars*) or into the cytoplasm (*open bars*) as described under "Experimental Procedures" either in the absence (*A*) or presence (*B*) of WGA. Average values of the percentage of injected cells positive for EGFP after 20 h are shown from three series of injections ± S.D.

dextran confined to the original compartment were included in the analysis, to exclude the effects of nuclear membrane breakdown on transfection. Direct injection of complexes into the nucleus resulted in over 70% frequency of EGFP expression for both PEI-DNA and mel-PEI-DNA complexes. Following intracytoplasmic injection of PEI-DNA complexes, only ~10% of cells showed positive EGFP signals. In contrast, however, mel-PEI-DNA complexes showed over 40% frequency of transgene expression following intracytoplasmic injection (Fig. 5*A*). To probe the mechanism of melittin-augmented transgene expression, complexes were co-injected with WGA, a lectin that binds *N*-acetyl-β-D-glucosamine and inhibits nuclear pore activity (25) (Fig. 5*B*). Intranuclear injection of WGA had no effect on the efficiency of expression of mel-PEI-DNA or PEI-DNA. In contrast, cytoplasmic injection of WGA decreased the transgene expression of mel-PEI-DNA complexes almost 4-fold, falling to levels similar to PEI-DNA, whereas the activity of PEI-DNA was essentially unaffected.

DISCUSSION

Melittin, the major component of bee venom, is a 26-amino acid cationic peptide that disrupts membranes and exhibits a strong cytotoxic activity (for reviews, see Refs. 13 and 26). In aqueous solution melittin forms amphipathic α-helices that interact with lipid membranes via a positively charged cluster (Lys-Arg-Lys-Arg) near the C terminus, inserting into the lipid bilayer and perturbing the structure. These activities, combined with its net positive charge, make melittin an interesting candidate for enhancing the delivery of DNA in transfection protocols. However, early attempts using unmodified melittin as a transfection agent showed only slightly enhanced transgene expression because of the fast partitioning of melittin into cell membranes, with concomitant high toxicity (27). Plank and colleagues (28) recently showed that a minimum number of seven positive charges per polycation is necessary to prevent disassembly of polyelectrolyte DNA complexes under physiological salt concentrations, hence, dissociation of DNA complexes with melittin, which has only five positive charges, may be anticipated.

One approach to improve the interaction of melittin with DNA has been to incorporate both molecules into cationic liposomes, by coupling melittin via an *N*-terminal cysteine to a neutral lipid moiety (29). The hybrid molecule dioleyl-melittin binds DNA via the cationic peptide, whereas the lipid component enhances hydrophobic collapse of the DNA. The resulting particles produced high transfection rates even in the presence of serum.

An alternative approach to increase the interaction of melittin with DNA is to link melittin to cationic polymers such as PEI, enabling formation of mel-PEI-DNA complexes. Whereas this is difficult with anionic peptides, because they can cause aggregation of positively charged PEI-DNA complexes (4, 11), the net positive charge of melittin enables formation of small and discrete mel-PEI-DNA complexes.

The possibility that melittin might endow mel-PEI-DNA complexes with increased membrane activity was examined using fluorescence microscopy, with fluorophores linked to both PEI and DNA. We also used a semiquantitative method described by Plank and colleagues, based on the ability of monensin to remove pH quenching of FITC fluorescence (4). Microscopy showed that the majority of cell-associated PEI-DNA complexes were contained within cytoplasmic vesicles, and the monensin assay indicated that these vesicles were effectively acidified. This is in accord with observations that transfection activity of PEI-DNA complexes can be enhanced by the presence of the endosomolytic agent chloroquine (11). In sharp contrast, fluorescence microscopic analysis of mel-PEI-DNA complexes showed a more dispersed fluorescence profile, whereas the monensin assay indicated that the complexes were not contained within a strong acid environment. Both observations are compatible with melittin mediating significant release of mel-PEI-DNA complexes into the cytoplasm.

The transfection activity of mel-PEI-DNA was evaluated against a broad range of cell types and cell lines, and showed good activity in each. The presence of the melittin mediated increases of 1–2 logs of gene expression in each case, compared with PEI-DNA complexes, and activity was independent of the presence of serum.[2] Formation of complexes in HBS to induce aggregation further promoted transgene expression with mel-PEI-DNA and was particularly useful on cells resistant to transfection such as IGROV1 cells. Primary cells that divide very slowly (*e.g.* HUVEC propagated in the absence of exogenous growth factors) or not at all (primary hepatocytes) are

[2] M. Ogris, R. C. Carlisle, T. Bettinger, and L. W. Seymour, unpublished observations.

almost completely resistant to PEI mediated transfection; however, the use of mel-PEI·DNA resulted in substantial increases in levels of reporter gene expression.

The profile of transgene expression obtained using mel-PEI·DNA not only showed 1–2 logs higher levels of transgene expression compared with PEI·DNA, but also showed expression at a much earlier time and reached a peak level several hours before PEI·DNA. Although this could simply reflect enhanced endosomal escape leading to earlier transcription of plasmid DNA, similar accelerated time courses of transgene expression have been reported previously for PEI-based systems that also contain a post-endosomal nuclear homing activity (8). This prompted us to consider the possibility that melittin endows nuclear homing activity on the PEI·DNA complexes, as well as its endosomolytic action.

Most nucleoproteins are targeted into the nucleus by NLS, typically composed of short amino acid sequences containing several positive charges. The primary structure of melittin contains just one region with characteristics similar to classical NLS, namely the KRKR sequence near the C terminus. In fact, this precise sequence has been implicated as having primary responsibility for mediating nuclear targeting of the protein dyskerin, although several other NLS on the protein synergize during import (30). Subramaniam and colleagues (31) showed that the ^{126}RKRKRSR^{132} sequence near the C terminus of interferon γ is responsible for its nuclear import in digitonin permeabilized cells (31), and, most recently, the sequence ^{196}KRKRR^{200} was described to be essential for the localization of a spliceosomal protein to nuclear speckles (32).

The possibility that enhanced nuclear localization might contribute to the activity of melittin-PEI·DNA complexes was assessed by measuring transfection in B16F10 cells with different growth rates and, hence, different levels of nuclear membrane integrity. As expected, melittin enhanced the activity of transfection in both types of cell. In rapidly proliferating cells, the presence of melittin appeared to remove the limiting effect of inefficient endosomal escape on transfection. However, in more quiescent cells, where overall levels of transfection are much lower, the activity of simple PEI·DNA complexes was limited by post-endosomal factors, whereas the greater activity of mel-PEI·DNA complexes was actually restricted by inadequate endosomal release. These observations support the possibility that melittin mediates a dual effect, combining endosomolysis with a post-endosomal activity, such as functional nuclear localization.

Microinjection of DNA vectors into the cytoplasm can give valuable information about NLS-mediated transport to the nucleus (17, 33, 34). In this study we found that microinjection of complexes into the nuclei of mammalian fibroblasts produced frequencies of transgene expression comparable with the injection of unmodified plasmids, regardless of the presence of melittin or WGA. Following intracytoplasmic injection, however, mel-PEI·DNA complexes showed 4-fold greater transgene expression frequency compared with PEI·DNA, demonstrating the ability of melittin to mediate a post endosomal effect. Co-injection of WGA inhibited this activity of melittin, decreasing transfection to levels shown by unmodified PEI·DNA complexes. This indicates that the post endosomal action of melittin involves the nuclear pore and fits with our hypothesis that melittin mediates NLS activity through its KRKR sequence.

In summary, the short peptide melittin possesses a dual endosomolytic and nuclear-homing functionality that can form the basis of a powerful transfection agent. Even the known immunogenicity of the naturally occurring L-melittin (35) can be overcome by using the synthetic D-form (36), and our studies have already confirmed that D-melittin-PEI shows the same beneficial activities for gene delivery.[2] Melittin-based polyelectrolyte transfection systems are likely to find several applications *in vitro*, and their small size and potential for combination with active targeting mechanisms make them interesting candidates for further development for systemic gene delivery *in vivo*.

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REFERENCES

1. Zabner, J., Fasbender, A. J., Moninger, T., Poellinger, K. A., and Welsh, M. J. (1995) *J. Biol. Chem.* **270**, 18997–19007
2. Tseng, W. C., Haselton, F. R., and Giorgio, T. D. (1999) *Biochim. Biophys. Acta* **1445**, 53–64
3. Erbacher, P., Roche, A. C., Monsigny, M., and Midoux, P. (1996) *Exp. Cell Res.* **225**, 186–194
4. Plank, C., Oberhauser, B., Mechtler, K., Koch, C., and Wagner, E. (1994) *J. Biol. Chem.* **269**, 12918–12924
5. Pollard, H., Remy, J. S., Loussouarn, G., Demolombe, S., Behr, J. P., and Escande, D. (1998) *J. Biol. Chem.* **273**, 7507–7511
6. Brunner, S., Sauer, T., Carotta, S., Cotten, M., Saltik, M., and Wagner, E. (2000) *Gene Ther.* **7**, 401–407
7. Sebestyen, M. G., Ludtke, J. J., Bassik, M. C., Zhang, G., Budker, V., Lukhtanov, E. A., Hagstrom, J. E., and Wolff, J. A. (1998) *Nat. Biotechnol.* **16**, 80–85
8. Zanta, M. A., Belguise-Valladier, P., and Behr, J. P. (1999) *Proc. Natl. Acad. Sci. U. S. A.* **96**, 91–96
9. Branden, L. J., Mohamed, A. J., and Smith, C. I. (1999) *Nat. Biotechnol.* **17**, 784–787
10. Subramanian, A., Ranganathan, P., and Diamond, S. L. (1999) *Nat. Biotechnol.* **17**, 873–877
11. Ogris, M., Steinlein, P., Kursa, M., Mechtler, K., Kircheis, R., and Wagner, E. (1998) *Gene Ther.* **5**, 1425–1433
12. Ogris, M., Brunner, S., Schuller, S., Kircheis, R., and Wagner, E. (1999) *Gene Ther.* **6**, 595–605
13. Dempsey, C. E. (1990) *Biochim. Biophys. Acta* **1031**, 143–161
14. Snyder, S. L., and Sobocinski, P. Z. (1975) *Anal. Biochem.* **64**, 284–288
15. Wagner, E., Cotten, M., Mechtler, K., Kirlappos, H., and Birnstiel, M. L. (1991) *Bioconjug. Chem.* **2**, 226–231
16. Ogris, M., Steinlein, P., Carotta, S., Brunner, S., and Wagner, E. (2000) *Biochim. Biophys. Acta* **1474**, 237–243
17. Carlisle, R. C., Bettinger, T., Ogris, M., Stuart, S., Mautner, V., Seymour, L. W. (2001) *Mol. Ther.*, in press
18. Dash, P. R., Read, M. L., Barrett, L. B., Wolfert, M. A., and Seymour, L. W. (1999) *Gene Ther.* **6**, 643–650
19. Geisow, M. J. (1984) *Exp. Cell Res.* **150**, 29–35
20. Maxfield, F. R. (1982) *J. Cell Biol.* **95**, 676–681
21. Murphy, R. F., Powers, S., Verderame, M., Cantor, C. R., and Pollack, R. (1982) *Cytometry* **2**, 402–406
22. Poulain, L., Ziller, C., Muller, C. D., Erbacher, P., Bettinger, T., Rodier, J. F., and Behr, J. P. (2000) *Cancer Gene Ther.* **7**, 644–652
23. Finsinger, D., Remy, J. S., Erbacher, P., Koch, C., and Plank, C. (2000) *Gene Ther.* **7**, 1183–1192
24. Kichler, A., Behr, J. P., and Erbacher, P. (1999) in *Nonviral Vectors for Gene Delivery* (Huang, L., Hung, M.-C., and Wagner, E., eds) pp. 191–206, Academic Press, San Diego
25. Yoneda, Y., Imamoto-Sonobe, N., Yamaizumi, M., and Uchida, T. (1987) *Exp. Cell Res.* **173**, 586–595
26. Kourie, J. I., and Shorthouse, A. A. (2000) *Am. J. Physiol.* **278**, C1063–C1087
27. Legendre, J. Y., and Szoka, F. C., Jr. (1993) *Proc. Natl. Acad. Sci. U. S. A.* **90**, 893–897
28. Plank, C., Tang, M. X., Wolfe, A. R., and Szoka, F. C., Jr. (1999) *Hum. Gene Ther.* **10**, 319–332
29. Legendre, J. Y., Trzeciak, A., Bohrmann, B., Deuschle, U., Kitas, E., and Supersaxo, A. (1997) *Bioconjug. Chem.* **8**, 57–63
30. Heiss, N. S., Girod, A., Salowsky, R., Wiemann, S., Pepperkok, R., and Poustka, A. (1999) *Hum. Mol. Genet.* **8**, 2515–2524
31. Subramaniam, P. S., Mujtaba, M. G., Paddy, M. R., and Johnson, H. M. (1999) *J. Biol. Chem.* **274**, 403–407
32. Eilbracht, J., and Schmidt-Zachmann, M. S. (2001) *Proc. Natl. Acad. Sci. U. S. A.* **98**, 3849–3854
33. Collas, P., Husebye, H., and Alestrom, P. (1996) *Transgenic Res.* **5**, 451–458
34. Vacik, J., Dean, B. S., Zimmer, W. E., and Dean, D. A. (1999) *Gene Ther.* **6**, 1006–1014
35. King, T. P., Coscia, M. R., and Kochoumian, L. (1993) *Biochemistry* **32**, 3506–3510
36. King, T. P., Wade, D., Coscia, M. R., Mitchell, S., Kochoumian, L., and Merrifield, B. (1994) *J. Immunol.* **153**, 1124–1131